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^{19}F-n.m.r. studies of 3′,5′-difluoromethotrexate binding to *Lactobacillus casei* dihydrofolate reductase

Molecular motion and coenzyme-induced conformational changes

G. Marius CLORE, Angela M. GRONENBORN, Berry BIRDSALL, James FEENEY and Gordon C. K. ROBERTS
Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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^{19}F-n.m.r. spectroscopy was used to study the binding of 3′,5′-difluoromethotrexate to dihydrofolate reductase (tetrahydrofolate dehydrogenase) from *Lactobacillus casei*. The benzoyl ring of the bound difluoromethotrexate was found to 'flip' about its symmetry axis, and the rate ($7.3 \times 10^3\,s^{-1}$ at 298 K) and activation parameters for this process were determined by lineshape analysis of the ^{19}F-n.m.r. spectrum at a series of temperatures in the range 273–308 K. The contributions to the barrier for this process are discussed. Addition of $NADP^+$ or NADPH to form the enzyme–difluoromethotrexate–coenzyme ternary complex led to an increase in the rate of benzoyl ring flipping by a factor of 2.6–2.8-fold, and to substantial changes in the ^{19}F-n.m.r. chemical shifts. The possible nature of the coenzyme-induced conformational changes responsible for these effects is discussed.

Methotrexate is an effective cytotoxic agent that acts by inhibiting the enzyme dihydrofolate reductase (tetrahydrofolate dehydrogenase, EC 1.5.1.3). Among the many derivatives of methotrexate that have been synthesized in the search for improved activity (for review see Roth & Cheng, 1982), those with halogen substituents on the benzoyl ring (Cosulich *et al.*, 1951; Angier *et al.*, 1959; Tomcufcik & Seeger, 1961; Marinelli & Chaykovsky, 1980) have been studied extensively. In particular, 3′,5′-dichloromethotrexate has been found to be useful in some clinical situations (Cleveland *et al.*, 1969; Vogel *et al.*, 1972).

We have been using n.m.r. spectroscopy to examine the binding of methotrexate and related inhibitors to dihydrofolate reductase (for references see Birdsall *et al.*, 1981; Roberts, 1983). However, in our previous studies it proved impossible to identify the benzoyl proton resonances of methotrexate bound to the enzyme. In the present paper, we show that, in complexes of 3′,5′-difluoromethotrexate (Fig. 1) with *Lactobacillus casei* dihydrofolate reductase, the ^{19}F-n.m.r. resonances of the bound ligand can be detected with ease and used to obtain information on the environment and dynamic properties of the benzoyl ring in the complex.

Experimental

Biochemical methods

Dihydrofolate reductase was purified from *Lactobacillus casei* MTX/R as described previously (Dann *et al.*, 1976). The purified enzyme was freeze-dried twice from ^2H_2O solution to remove most of the exchangeable protons and then redissolved to give an approx. 1 mM enzyme solution in ^2H_2O containing 50 mM-potassium phosphate/500 mM-KCl/1 mM-EDTA, pH* 6.5 (meter reading uncorrected for the isotope effect on the glass electrode). Dioxan (1 mM) was added as a ^{1}H-n.m.r. chemical-shift reference; its resonance is 3.71 p.p.m. downfield of 4,4-dimethylsilapentane-1-sulphonate.

3′,5′-Difluoromethotrexate, prepared by the method of Tomcufcik & Seeger (1961), was a gift from Dr. D. G. Johns (National Cancer Institute, Bethesda, MD, U.S.A.). The sample contained about 20% of an impurity identified as the starting material *N*-(3,5-difluoro-4-methylaminobenzoyl)-L-glutamate. Pure samples of the enzyme–difluoromethotrexate complex, free of this impurity, were prepared as follows. To a solution containing approx. 1 mM enzyme was added sufficient of the impure difluoromethotrexate sample to ensure that

the concentration of difluoromethotrexate itself was as nearly as possible equal to that of the enzyme. Since difluoromethotrexate has a binding constant greater than $10^9 M^{-1}$, whereas *N*-(3,5-difluoro-4-methylaminobenzoyl)-L-glutamate would be expected to have a binding constant less than $10^4 M^{-1}$ (Dann *et al.*, 1976; Birdsall *et al.*, 1978; B. Birdsall, unpublished work), this solution will consist of the enzyme–difluoromethotrexate complex and free *N*-(3,5-difluoro-4-methylaminobenzoyl)-L-glutamate. The latter was removed by dialysis against the phosphate/KCl buffer described above.

$NADP^+$ and NADPH were obtained from Sigma Chemical Co. and used without further purification. Ternary complexes were formed by adding 2 molar equivalents of $NADP^+$ or NADPH to a solution of the enzyme–difluoromethotrexate complex.

N.m.r. spectroscopy

^{19}F-n.m.r. spectra were recorded at 188.3 MHz with a Bruker WM 200 spectrometer. A spectral width of 10 kHz was used, and 16384 data points were collected, with quadrature detection; 4000 transients were averaged for each spectrum. ^{1}H decoupling was not employed, so that unresolved ^{19}F–^{1}H spin-coupling makes a contribution of up to 9 Hz to the observed linewidths. Spectra were obtained at temperatures in the range 273–313 K (temperature controlled to ± 1 K with a stream of dry compressed air); ^{1}H-n.m.r. spectra showed clearly that the enzyme complex remained in its native conformation over this range.

^{1}H-n.m.r. spectra were recorded at 270 MHz with a Bruker WH 270 spectrometer. A spectral width of 4.2 kHz was used, and 8192 data points were collected, with quadrature detection; 1000–2000 transients were averaged for each spectrum. pH titrations were carried out as described previously (Birdsall *et al.*, 1977; Wyeth *et al.*, 1980).

Lineshape analysis

The lineshapes of the ^{19}F-n.m.r. resonances were analysed by using the following equations for exchange between two equally populated sites (see Pople *et al.*, 1959; Emsley *et al.*, 1965), based on McConnell's (1958) modification of the Bloch equations:

$$g(\nu) = S \cdot \frac{(1 + \pi\Delta/k)\,P + QR}{P^2 + R^2} \qquad (1)$$

where

$$P = 4\pi^2[k^{-1}(\Delta^2/4 - \nu^2 + \delta^2/4) + \Delta/4\pi] \qquad (2)$$

$$Q = -2\pi\nu/k \qquad (3)$$

$$R = -2\pi\nu(1 + 2\pi\Delta/k) \qquad (4)$$

ν is the frequency, expressed relative to $\frac{1}{2}(\nu_A + \nu_B)$, where ν_A and ν_B are the resonance frequencies of the nucleus of interest in the two sites A and B; $\delta = \nu_A - \nu_B$; Δ is the linewidth of the resonance in the absence of exchange (assumed to be the same in both sites); S is a scaling factor. k, the rate of exchange between the sites (i.e. the sum of the forward and reverse rate constants, which are equal), is given at any temperature T by:

$$k_T = k_{298} \cdot \exp\left[E_A R^{-1}\left(\frac{1}{298} - \frac{1}{T}\right)\right] \qquad (5)$$

where 298 K is used as a reference temperature, and E_A is the activation energy for the exchange process.

The experimental lineshapes at eight temperatures were fitted simultaneously to eqns. (1) and (5) by optimizing the four parameters k_{298}, E_A, δ and S. (In initial optimizations, Δ was also optimized, but it was found to be poorly determined, and so it was held constant at a value of 30 Hz in subsequent optimizations; this value includes 20 Hz linebroadening introduced to improve the signal-to-noise ratio.) The chemical-shift difference, δ, was assumed to be temperature-independent; support for this assumption comes from the observation that the average chemical shift, $\frac{1}{2}(\nu_A + \nu_B)$, was only very slightly temperature-dependent. Optimization was carried out by using the FACSIMILE program (Chance *et al.*, 1977) and the fitting procedures described previously (Clore & Chance, 1978; Clore, 1983).

Results and discussion

Comparison of methotrexate and difluoromethotrexate binding

The chemical shifts of the C2-proton resonances of the seven histidine resonances of *L. casei* dihydrofolate reductase in its complex with difluoromethotrexate have been measured as a function of pH between pH* 5.5 and 8.5. Within experimental error, each resonance showed behaviour identical with that reported previously for the enzyme–methotrexate complex (Gronenborn *et al.*, 1981). In particular, in both complexes the pK of His-28 is increased by approx. 1 unit relative to its value in the free enzyme. This increase in pK arises from an ion-pair interaction between the γ-carboxylate group of methotrexate and the imidazole ring of His-28 (Matthews *et al.*, 1978; Bolin *et al.*, 1982). We have recently shown (Antonjuk *et al.*, 1983) that this interaction of the γ-carboxylate group of methotrexate depends, in turn, on the interaction of the α-carboxylate group with Arg-57. The observation of an increased pK of His-28 thus indicates that the glutamate moiety of difluoromethotrexate

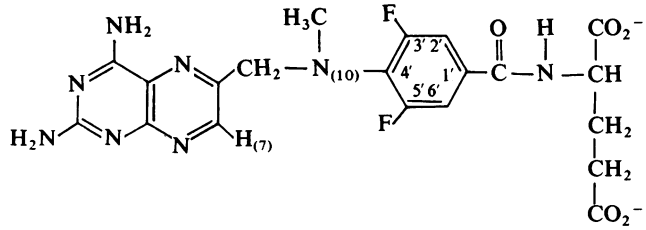

Fig. 1. *Structure of 3',5'-difluoromethotrexate*

binds to the enzyme in the same way as the corresponding part of methotrexate.

Since difluoromethotrexate retains a high affinity for the enzyme ($>10^9 M^{-1}$; B. Birdsall, unpublished work) and the pteridine ring makes the major contribution to the overall binding energy (Roberts, 1983, and references cited therein), it seems likely that this part of the molecule will also bind in a similar fashion to that observed for methotrexate in the crystal (Matthews *et al.*, 1978; Bolin *et al.*, 1982). However, whereas the pteridine 7-proton resonance of methotrexate bound to the enzyme appears at 4.15p.p.m. (Feeney *et al.*, 1977; Wyeth *et al.*, 1980), the corresponding signal from bound difluoromethotrexate is found to be 0.29p.p.m. farther downfield at 4.44p.p.m. The chemical shift of this resonance is influenced by the magnetic anisotropy of three nearby aromatic rings (Phe-30, Phe-49 and the benzoyl ring of the methotrexate molecule itself) and will be very sensitive to slight differences in orientation or conformation of the bound inhibitor. Indeed, in aminopterin, the N^{10}-desmethyl analogue of methotrexate, which behaves in all other respects very much like methotrexate, the 7-proton resonance of the bound inhibitor appears at 4.49p.p.m. (G. C. K. Roberts, J. Feeney & B. Birdsall, unpublished work), close to the position observed in difluoromethotrexate.

It seems likely, therefore, that difluoromethotrexate binds to dihydrofolate reductase in a way closely similar to, if not identical with, the mode of binding of methotrexate itself.

^{19}F-n.m.r. spectroscopy

Fig. 2 shows the ^{19}F-n.m.r. spectra of the difluoromethotrexate–dihydrofolate reductase complex at a series of temperatures between 273K and 308K. At 308K a single resonance was observed from both fluorine nuclei, 1.04p.p.m. downfield from the resonance of free difluoromethotrexate. As the temperature was lowered the signal broadened, and below 283K two separate resonances of equal intensity were observed, which became narrower as the temperature was lowered further (see Fig. 2). These observations indicate that the 3'- and 5'-fluorine nuclei experience the same environment at high temperatures but different environments at low temperatures. The ^{19}F-n.m.r. spectra shown in Fig. 2 are characteristic of nuclei undergoing exchange between two equally populated magnetically non-equivalent sites. Because of the asymmetric nature of the binding site, any fixed orientation of the benzoyl ring would be expected to result in magnetic non-equivalence of the 3'- and 5'-fluorine nuclei. The observed spectra could be explained if there is an exchange process that has the effect of interchanging the positions of the 3'- and 5'-fluorine atoms so that at high temperatures they experience an average environment. The simplest such exchange process would be motion about the symmetry axis of the benzoyl ring, i.e. simultaneous rotation about the $N_{(10)}$–$C_{(4')}$ and $C_{(1')}$–CO bonds. Similar motions about the symmetry axes of aromatic rings have been detected for phenylalanine and tyrosine residues in a number of proteins (Campbell *et al.*, 1975, 1976; Snyder *et al.*, 1975; Wüthrich & Wagner, 1975; Wagner, 1980; Karplus & McCammon, 1981) as well as for ligands bound to proteins (Cayley *et al.*, 1979; Feeney *et al.*, 1981). The ^{19}F-n.m.r. spectra alone cannot distinguish between free rotation and 180° jumps about the symmetry axis. Several lines of evidence suggest that the latter model, involving a 'flipping' motion, is substantially more probable (Hetzel *et al.*, 1976; Karplus & McCammon, 1981; Gall *et al.*, 1982; Wagner, 1983). In the present case, both the $N_{(10)}$–$C_{(4')}$ an d $C_{(1')}$–CO bonds of difluoromethotrexate have significant double-bond character, which would favour a twofold potential for rotation about these bonds.

We have thus fitted the ^{19}F-n.m.r. spectra (at all eight temperatures simultaneously) with lineshapes calculated assuming exchange between two equally populated sites, by using procedures described in the Experimental section. The best-fit theoretical spectra are shown alongside the experimental spectra in Fig. 2. The rate of the 'flipping' process, the activation parameters and the ^{19}F-n.m.r. chemical shifts derived from the optimization are given in Table 1. It should be noted that all

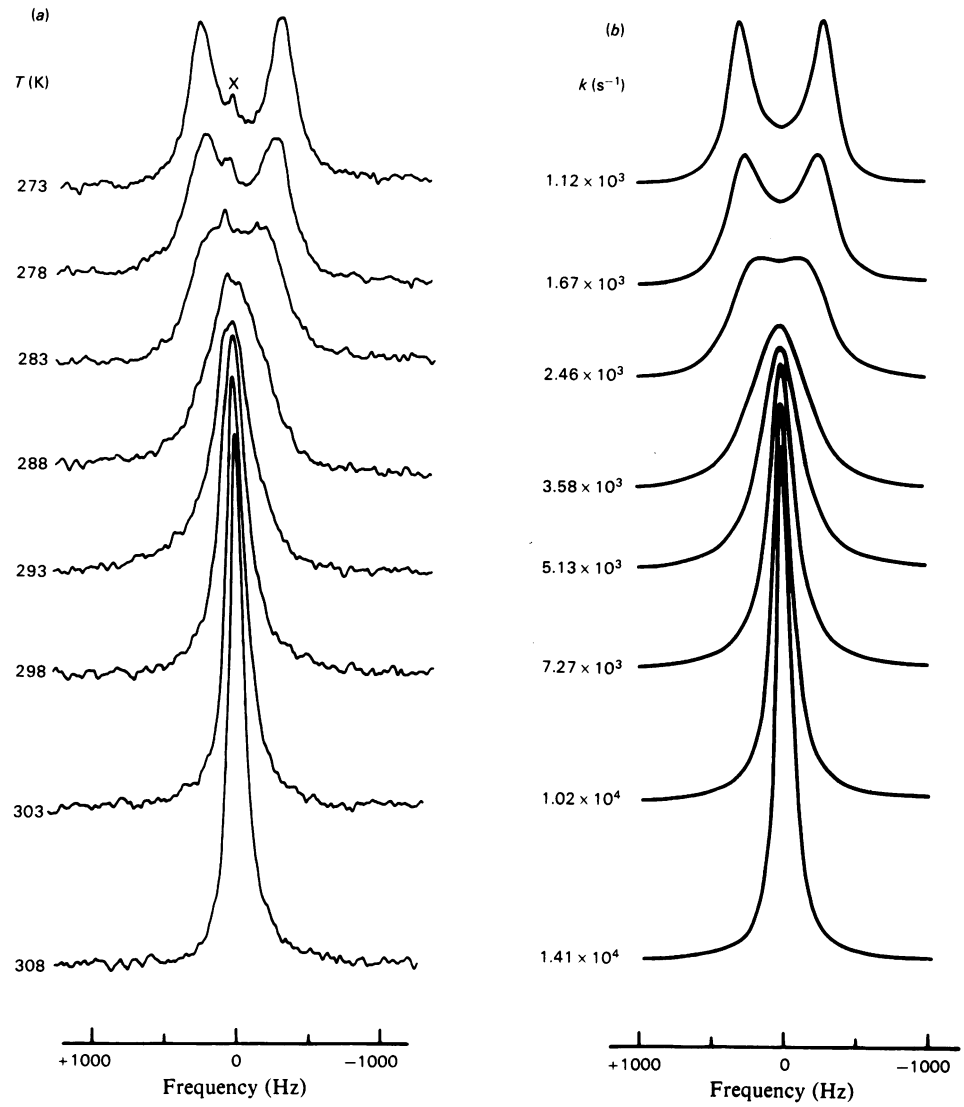

Fig. 2. *Observed (a) and calculated (b) 188.3 MHz ^{19}F-n.m.r. spectra of 3',5'-difluoromethotrexate bound to L. casei dihydrofolate reductase*

The sample contained slightly less than 1 molar equivalent of difluoromethotrexate, so that all the ligand is bound to the enzyme. The small sharp resonance marked X represents a small amount (<5%) of the impurity *N*-(3',5'-difluoro-4-methylaminobenzoyl)-L-glutamate. The calculated spectra were derived from the best-fit parameters in Table 1. For each pair of experimental and calculated spectra the experimental temperature and 'best-fit' rate constant (from the parameters in Table 1) are indicated. The frequency axis is referred to the averaged bound difluoromethotrexate resonance as zero.

the parameters are exceptionally well determined with relative errors of about ±1% or less, with the exception of the value for the entropy of activation, which, though still well determined, has a relative error of about ±15%. This good determination of the parameters was only possible because the data at all temperatures were fitted simultaneously; if the data at each temperature had been fitted individually the values of the parameters could not have been well defined. At 298K, the rate of 'flipping' about the $N_{(10)}$–$C_{(4')}$ and $C_{(1')}$–CO bonds is 7.3 $10^3 s^{-1}$, and the free energy of activation is 52.7 kJ/mol. The entropy of activation is small so that the activation enthalpy is very similar to the activation free energy.

The barrier to the 'flipping' process will be made

Table 1. *Rate of 'flipping', the activation parameters and the ^{19}F-n.m.r. chemical shifts of 3',5'-difluoromethotrexate bound to dihydrofolate reductase*

The values were obtained by lineshape analysis of the ^{19}F-n.m.r. spectra at eight temperatures, fitted simultaneously as described in the text. The overall standard deviation of the fit was in all cases $\leq 5\%$, which is within the overall standard error of the data ($\sim 5\%$), and the distribution of residuals was random.

Complex ...	Dihydrofolate reductase–difluoromethotrexate	Dihydrofolate reductase–difluoromethotrexate–$NADP^+$	Dihydrofolate reductase–difluoromethotrexate–NADPH
k_{298} (s^{-1})*	$7.27 (\pm 0.07) \times 10^3$	$1.88 (\pm 0.04) \times 10^4$	$2.04 (\pm 0.01) \times 10^4$
E_A (kJ/mol)*	50.6 (±0.4)	43.0 (±0.4)	43.9 (±0.4)
$\Delta G^{\ddagger}_{298}$ (kJ/mol)†	52.7 (±0.03)	50.3 (±0.05)	50.1 (±0.08)
$\Delta H^{\ddagger}_{298}$ (kJ/mol)†	48.1 (±0.4)	40.5 (±0.4)	41.4 (±0.4)
$\Delta S^{\ddagger}_{298}$ ($J \cdot K^{-1} \cdot mol^{-1}$)†	−15 (±2)	−33 (±2)	−29 (±2)
δ (Hz)*	637 (±3)	1070 (±11)	746 (±15)
Chemical shifts (p.p.m.)‡	+2.73 −0.65	+3.54 −2.14	+2.86 −1.10

* Optimized parameter; see the Experimental section.

† Calculated from the optimized parameters. Errors were calculated in accordance with Clore & Chance (1978).

‡ Calculated from the optimized δ and the measured average shift, and expressed relative to free 3',5'-difluoromethotrexate.

up of two components: the 'intrinsic' barrier to rotation about the $N_{(10)}$–$C_{(4')}$ and $C_{(1')}$–CO bonds and the additional barrier due to the sterically restricted environment of the binding site. Although an exact model is not available, an approximate estimate of the 'intrinsic' barrier can be obtained from low-temperature n.m.r. studies of *p*-(dimethylamino)benzylaldehyde and *p*-(dimethylamino)acetophenone (Grindley *et al.*, 1975; Drakenberg *et al.*, 1980; and references cited therein). The activation enthalpy for rotation about the N–Ar bond is approx. 30kJ/mol, whereas for rotation about the Ar–CO bond $\Delta H^{\ddagger}$ is approx. 44kJ/mol for the benzaldehyde and $\Delta H^{\ddagger}$ is approx. 34kJ/mol for the acetophenone. The barrier to rotation about the Ar–CO bond in a benzamide would be expected to be somewhat lower than in a benzaldehyde or acetophenone (Hehre *et al.*, 1972), since the amide group is not completely co-planar with the aromatic ring (cf. procainamide; Peeters *et al.*, 1980) and this decreases the double-bond character of the Ar–CO bond. However, it is clear that the intrinsic barriers to rotation about the $N_{(10)}$–$C_{(4')}$ and $C_{(1')}$–CO bonds of difluoromethotrexate must make up a considerable part (more than two-thirds) of the observed $\Delta H^{\ddagger}$ of 48kJ/mol.

To identify the interactions with the protein that would contribute to the remainder of the barrier, we have examined the refined crystal structures of the *L. casei* and *Escherichia coli* enzymes (Bolin *et al.*, 1982). The protein residues were held in fixed positions while the benzoyl ring of the bound methotrexate was rotated in 30° steps about the $N_{(10)}$–$C_{(4')}$–$C_{(1')}$–CO axis, and the distances between the benzoyl-ring hydrogen atoms and the non-hydrogen atoms of nearby amino acid residues were calculated for each orientation. In Table 2 we summarize those distances less than 0.25nm (2.5Å), i.e. less than the sum of the van der Waals radii and representing substantially unfavourable steric interactions.

Table 2. *Distances between the benzoyl hydrogen atoms of methotrexate and neighbouring amino acid residues of dihydrofolate reductase as a function of rotation of the benzoyl ring*

Distances were calculated from co-ordinates of the refined crystal structures of the *L. casei* and *E. coli* enzymes (Bolin *et al.*, 1982). Only the results from the *L. casei* enzyme–methotrexate–NADPH complex are given; those for the *E. coli* enzyme–methotrexate complex were generally similar (in the *E. coli* enzyme the corresponding residue to Phe-49 is Ile-49). Only distances between benzoyl hydrogen atoms and non-hydrogen atoms of amino acid residues less than 0.25nm (2.5Å) are given. The torsion angle is expressed with the value in the crystal structure taken as zero, at which there were no contacts with $r < 0.25$nm (2.5Å).

$N_{(10)}$–$C_{(4')}$ torsion angle	Residue (atom)	Distance (nm)
30°	Phe-49 ($C_{(\varepsilon 1)}$)	0.234 (2.34Å)
	Leu-54 ($C_{(\delta 1)}$)	0.242 (2.42Å)
60°	Phe-49 ($C_{(\delta 1)}$)	0.174 (1.74Å)
	Phe-49 ($C_{(\varepsilon 1)}$)	0.171 (1.71Å)
	Leu-54 ($C_{(\delta 1)}$)	0.249 (2.49Å)
90°	Leu-27 ($C_{(\delta 2)}$)	0.208 (2.08Å)
	Phe-49 ($C_{(\delta 1)}$)	0.154 (1.54Å)
	Phe-49 ($C_{(\varepsilon 1)}$)	0.224 (2.24Å)
120°	Phe-49 ($C_{(\alpha)}$)	0.246 (2.46Å)
	Phe-49 ($C_{(\delta 1)}$)	0.247 (2.47Å)
	Pro-50 ($C_{(\delta)}$)	0.235 (2.35Å)
150°	Pro-50 ($C_{(\delta)}$)	0.204 (2.04Å)

It is clear from Table 2 that rotation of the

benzoyl ring about its symmetry axis within a rigid binding site would be accompanied by severe steric hindrance and hence a very large barrier to rotation. The rather low estimated contribution of the protein to the observed barrier ($\leqslant$20kJ/mol) must imply that the benzoyl-ring-binding site is not rigid. A number of theoretical studies (Gelin & Karplus, 1975; Hetzel *et al.*, 1976; McCammon *et al.*, 1983; Wagner, 1983) of the closely analogous 'flipping' of the aromatic rings of tyrosine and phenylalanine residues in proteins have shown that the low observed barriers can only be accounted for by 'relaxation' of the protein, i.e. small transient displacements of atoms near the ring. Similar fluctuations in the positions of nearby amino acid residues must account for the relatively low barrier to 'flipping' of the benzoyl ring of bound difluoromethotrexate. It can be seen from Table 2 that among the atoms potentially making close contact with the ring is the $C_{(\alpha)}$ atom of Phe-49. This suggests that these positional fluctuations involve the polypeptide backbone as well as the side chains (see also McCammon *et al.*, 1983).

The chemical shifts of the two ^{19}F nuclei on opposite sides of the benzoyl ring differ by 3.38p.p.m. (Table 1). The two major environmental contributions to ^{19}F-n.m.r. chemical shifts are likely to be second-order electric-field effects ('van der Waals' shifts; Feeney *et al.*, 1966), which produce downfield shifts, and magnetic-anisotropy effects (e.g. from neighbouring aromatic rings), which can produce either upfield or downfield shifts. The observation that one of the ^{19}F-n.m.r. signals of difluoromethotrexate shifts upfield 0.65p.p.m. on binding suggests that in this case there is a substantial contribution from magnetic-anisotropy effects. In the crystal structure (Bolin *et al.*, 1982), one of the 3',5'-hydrogen atoms is relatively close to the ring of Phe-30, and we calculate that this will produce an upfield shift of 0.95p.p.m. It seems likely that the higher-field ^{19}F-n.m.r. resonance in Fig. 2 arises from the corresponding fluorine atom.

Effects of coenzyme binding

Similar ^{19}F-n.m.r. experiments have been carried out on the ternary complexes enzyme–difluoromethotrexate–$NADP^+$ and enzyme–difluoromethotrexate–NADPH. In both cases the spectra were generally similar to those of the binary complex shown in Fig. 2, and were clearly characteristic of exchange between two equally populated sites. Lineshape analysis was carried out as described above, and the rates of 'flipping', the activation parameters and the chemical shifts are given in Table 1. The determination of these parameters for the ternary complexes is as good as those for the binary complex, so that all differences between the binary and ternary complexes are significant. The rate of 'flipping' of the benzoyl ring is seen to be increased by a factor of 2.6–2.8-fold in the presence of the coenzymes. Examination of the activation parameters, which are very similar in both ternary complexes, shows that the decrease in free energy of activation (at 298K) of 2.5kJ/mol arises from a decrease in $\Delta H^{\ddagger}$ of about 7kJ/mol, partially compensated by a substantially more negative activation entropy. In addition, the coenzymes change the ^{19}F-n.m.r. chemical shifts of bound difluoromethotrexate. By contrast with their very similar effects on the 'flipping' process, the oxidized and the reduced coenzymes have clearly different effects on the chemical shifts. NADPH affects predominantly the higher-field signal (tentatively assigned to the fluorine atom closer to Phe-30), but $NADP^+$ produces shifts of both resonances, leading to a substantial increase in the chemical-shift difference between the fluorine nuclei on opposite sides of the benzoyl ring.

The observation that addition of coenzymes to the enzyme–difluoromethotrexate complex leads to an increase in the rate of 'flipping' of the benzoyl ring clearly indicates that coenzyme binding leads to some change in the protein conformation in the vicinity of the benzoyl-ring-binding site.

Comparison of the refined crystal structures of the *L. casei* enzyme–methotrexate–NADPH and *E. coli* enzyme–methotrexate complexes (Bolin *et al.*, 1982) reveals one significant difference in the benzoyl-ring environment between the binary and the ternary complexes. In the binary complex of the *E. coli* enzyme, a nitrogen atom of the guanidinium group of Arg-52 forms a hydrogen bond to the benzoyl carbonyl oxygen atom, but in the ternary complex of the *L. casei* enzyme the benzoyl carbonyl oxygen atom does not form a hydrogen bond to the protein, and the orientation of the Arg-52 side chain is quite different (Bolin *et al.*, 1982). It is not certain from this comparison alone that these differences can be ascribed to a conformational change produced by coenzyme binding, since the sequence of the *L. casei* enzyme contains a one-residue insertion relative to that of the *E. coli* enzyme immediately preceding Arg-52 (Volz *et al.*, 1982, and references cited therein). The ^{19}F-n.m.r. data, however, demonstrate clearly that the coenzyme does change the conformation of this region of the protein molecule. Such a change in the interaction of the benzoyl carbonyl group with the enzyme might lead to faster 'flipping' of the benzoyl ring if the benzamide were made less planar, thus decreasing the double-bond character of the Ar–CO bond, as discussed above. The available crystallographic co-ordinates of the two complexes are not sufficiently precise to allow us to test this possibility. Alternatively, coenzyme binding

might lead to a slight movement of, for example, Phe-49 and Pro-50 away from the benzoyl ring, so as to decrease the protein contribution to the rotational barrier. Even quite small movements of these residues would lead to a significant decrease in the barrier. One possibility is that the interactions of Arg-43, Arg-44 and Thr-45 with the coenzyme might lead to a slight lateral movement of helix C (residues 42–49).

Although the binding of coenzyme increases the rate of 'flipping', it also increases the affinity of the enzyme for both methotrexate (Birdsall *et al.*, 1980*a*) and *p*-aminobenzoylglutamate (Birdsall *et al.*, 1978, 1980*b*). Since the interaction of the glutamate γ-carboxylate group with His-28. is not affected by the presence of coenzyme (Gronenborn *et al.*, 1981), it is likely that the co-operativity in binding between coenzyme and *p*-aminobenzoylglutamate originates in the benzoyl-ring-binding site; the oxidized and the reduced coenzymes have the same effect (Birdsall *et al.*, 1980*b*). The same conformational change may be involved in both the increased binding energy of the benzoyl ring and its increased rate of 'flipping'

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